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Regulation of neuronal high-voltage activated Ca_V2 Ca^{2+} channels by the small GTPase RhoA

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ABSTRACT

High-Voltage-Activated (HVA) Ca^{2+} channels are known regulators of synapse formation and transmission and play fundamental roles in neuronal pathophysiology. Small GTPases of Rho and RGK families, via their action on both cytoskeleton and Ca^{2+} channels are key molecules for these processes. While the effects of RGK GTPases on neuronal HVA Ca^{2+} channels have been widely studied, the effects of RhoA on the HVA channels remains however elusive. Using heterologous expression in *Xenopus laevis* oocytes, we show that RhoA activity reduces Ba^{2+} currents through $Ca_V2.1$, $Ca_V2.2$ and $Ca_V2.3$ Ca^{2+} channels independently of $Ca_V\beta$ subunit. This inhibition occurs independently of RGKs activity and without modification of biophysical properties and global level of expression of the channel subunit. Instead, we observed a marked decrease in the number of active channels at the plasma membrane. Pharmacological and expression studies suggest that channel expression at the plasma membrane is impaired via a ROCK-sensitive pathway. Expression of constitutively active RhoA in primary culture of spinal motoneurons also drastically reduced HVA Ca^{2+} current amplitude. Altogether our data revealed that HVA Ca^{2+} channels regulation by RhoA might govern synaptic transmission during development and potentially contribute to pathophysiological processes when axon regeneration and growth cone kinetics are impaired.

1. Introduction

Calcium influx via neuronal voltage-gated Ca^{2+} channels (VGCC) plays a key role in a number of neuronal functions including secretion, synaptic transmission, neuronal development, cell proliferation and survival, and gene expression. These channels transduce neuronal electrical activity into a timely-, spatially-resolved and cell-type specific intracellular signal. Ten genes have been identified in the mammalian genome encoding the pore-forming VGCC subunits that display distinct biophysical and pharmacological properties. Only those of the Ca_V2 family ($Ca_V2.1$, $Ca_V2.2$ and $Ca_V2.3$) are mainly neuronal and are expressed at specific pre- and post-synaptic sites. Indeed, impaired channel function, as well as several genetic mutations in channel subunits has been associated with pathophysiological situations including epilepsy, hemiplegia, ataxia, neuro-degeneration and spinal muscular atrophy (Simms and Zamponi, 2014; Jablonka et al., 2007).

VGCC share the prototypic multimeric structure common to all Ca_V1 and Ca_V2 Ca^{2+} channels, which encompass a pore-forming subunit encoded by the $Ca_V\alpha1$ gene and two auxiliary $Ca_V\alpha2$-δ and

Abbreviations: HVA, high-voltage activated; LVA, low-voltage activated; VGCC, voltage-gated calcium channel; RGK GTPase, Rem Gem Kir GTPase; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic facto; CNTF, Ciliary neurotrophic factor; SMA, spinal muscular atrophy; HEK cells, human embryonic cells; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BAPTA, 1,2-bis(o-amino phenoxy)ethane-N,N,N′,N'-tetraacetic acid.

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$Ca_V\beta$ subunits for which four genes for each are known. The transmembrane $Ca_V\alpha1$ subunit represents the pharmacologically defined Ca^{2+} channel with expression and biophysical properties regulated by the juxtamembranar $Ca_V\alpha2$-δ and the intracellular $Ca_V\beta$ subunits. VGCC are also the target of multiple regulatory pathways that modify their biophysical properties (voltage-dependency, gating kinetics or open probability), expression levels and subcellular localization.

In the recent past, regulation of Ca^{2+} channel expression and activity by small GTPases has been the subject of intense experimental work. Binding of the RGK GTPase Gem to $Ca_V\beta$ was first revealed by a yeast-two-hybrid screen, and their interaction was found to completely block the $Ca_V1.2$ L-type Ca^{2+} channel currents (Beguin et al., 2007). It was subsequently found that all members of the RGK family (Gem, Rem, Rem2 and Rad) were able to produce this inhibition, which was extended to other HVA channels ($Ca_V1.2$, $Ca_V1.3$, $Ca_V2.1$, $Ca_V2.2$ and $Ca_V2.3$) (Leyris et al., 2009; Yang and Colecraft, 2012; Charnet et al., 2013; Flynn and Zamponi, 2010). In contrast, LVA channels are completely insensitive to RGK inhibition (Finlin et al., 2003; Chen et al., 2005; Fan et al., 2010). RGK binding to the $Ca_V\beta$ subunit was absolutely required to inhibit Ca^{2+} current. However, secondary binding-sites of RGKs on the pore-forming $Ca_V\alpha$ subunit ($Ca_V1.2$ or $Ca_V2.1$) were also involved in the inhibition of channel expression at the membrane and the decrease probability of channel opening (Fan et al., 2010; Yang et al., 2012; Fan et al., 2012; Pang et al., 2010).

More recently, Ca^{2+} current inhibition by the RhoA small GTPase was reported (Iftinca et al., 2007; Piccoli et al., 2004; Marrs et al., 2009). RhoA promotes cytoskeletal rearrangement and stress fiber assembly through its primary effector the Rho-associated protein kinase (ROCK), for which two genes, ROCK I/β and ROCK II/α,are present in mammals (Ridley, 1997). Similar to all small GTPases, RhoA cycles between an active (GTP-bound) and an inactive (GDP-bound) state. In neurons, RhoA (RhoA-GTP) activation participates in membrane trafficking, dendritic retraction, axon pathfinding and synaptogenesis (Luo et al., 1997). Indeed, mice overexpressing a dominant negative form of RhoA have reduced muscle innervation, defective neuronal patterning and decreased number of motoneurons (Kobayashi et al., 2004, 2004, 2011). Moreover the RhoA/ROCK pathway appears to contribute to pathogenesis of the motoneuron disease, spinal muscular atrophy (SMA). Functionally active RhoA and ROCK are observed in the spinal cord of SMA mice as well as SMA neuronal-like cells (Bowerman et al., 2010, 2007; Nolle et al., 2011). Importantly, ROCK inhibitors demonstrate significant therapeutic benefits in SMA mice (Bowerman et al., 2010, 2012), and, therefore, they have been proposed as therapeutic candidates to improve axon regeneration and neuronal survival (Coque et al., 2014). In dorsal root ganglion neurons (DRGs) and the spinal dorsal horn, chronic treatment with lysophosphatidic acid (LPA) activates RhoA, and increases VGCC expression and currents, producing an allodynia-type pain (Ogawa et al., 2012), which is reversed by the $Ca_V\alpha2$-δ modulator gabapentine. RhoA/ROCK signalling also regulates the trafficking of ion channels (Stirling et al., 2009; Liu et al., 2012; Pochynyuk et al., 2007) and decrease low-voltage-activated (LVA) Ca^{2+} currents via the phosphorylation of the I-II loop of Ca_V3 subunit (Iftinca et al., 2007). The regulation of the HVA Ca_V1 and Ca_V2 channels by the RhoA GTPases however, has been much less characterized so far and remains controversial (Yatani et al., 2005; Iftinca et al., 2007). Therefore, altogether, these considerations suggest that RhoA GTPase could contribute to the regulation of Ca^{2+} current, however, the mechanisms and the specificity of this regulation toward neuronal Ca_V2 channels remain unclear.

In this study, we show that activation of RhoA promotes Ca^{2+} channel inhibition through the pore-forming $Ca_V2.1$, $Ca_V2.2$ and $Ca_V2.3$ subunits. The RhoA-mediated inhibition of $Ca_V\alpha1$ activity appeared to occur in a Gem-independent manner. This inhibition was independent of $Ca_V\beta$ subunit expression but rather involved activation of ROCK and a subsequent decrease in the number of active channels at the plasma membrane. Moreover, we provide evidences that in primary motoneurons RhoA led to decrease Ca^{2+} current amplitude via HVA Ca^{2+} channels inhibition. This pathway may therefore play a role in pathological situations in which a decrease in neuronal excitability is observed together with the activation of the RhoA pathway such as SMA or other motoneurons diseases (Jablonka et al., 2007; Tarabal et al., 2014).

2. Materials and methods

This study was carried out in strict accordance with the recommendations of our institution. The protocol was approved by the *Direction départementale de la protection des populations* (Permit Number: C34-16). Surgery was performed under anesthesia, and efforts were made to minimize suffering. The care and use of mice conformed to institutional policies and guidelines. Mice were housed in cages with a 12 h light/12 h dark cycle, with food and water supplied *ad libitum*.

2.1. RNA preparation and oocyte injection

Xenopus oocytes were isolated from anesthetized *Xenopus leavis* as already described (Leyris et al., 2009), and injected in the equatorial region with 40–60 nl of deionised water containing 1 μg/μl of Ca^{2+} channel subunit RNAs synthetized *in vitro*. The RNA mixture contained rabbit $Ca_V2.1$, $Ca_V2.2$ or $Ca_V2.3$, $Ca_V\alpha_2\delta1$ and $Ca_V\beta_2$ or $Ca_V\beta_4$ Ca^{2+} channel subunits RNA at a W:W ratio of 1:1:1. This mixture was diluted at 1:1 with either deionised water or a solution containing the dominant active form of RhoA (Da-RhoA = RhoA-Q63L), or ROCK1 (ROCK1Δ1) RNA (at 1 μg/μl). The following cDNAs were used $Ca_V2.1$: Genbank X57476, $Ca_V2.2$: Genbank D14157, $Ca_V2.3$: Genbank X67855, $Ca_V\beta_2$: Genbank M80545, $Ca_V\beta_4$: Genbank L02315, $Ca_V\alpha_2\delta_1$: Genbank M86621, ROCK1: Genbank BC113114, RhoAQ63L from Cytoskeleton Inc. (Denver CO). A stop codon was introduced in WT ROCK1 after the codon encoding L_{1111} to give ROCK1Δ1. Gem was cloned from human expressed sequence tag IMAGE clone ID 4823985 (Leyris et al., 2009). cDNAs were linearized and cRNAs were *in vitro* transcribed using the mMESSAGE mMACHINE transcription kits (Life Technologies) according to the manufacturer's instructions.

Channel expression was tested 2–5 days later. Electrophysiological recordings were performed the same day for control and RhoA/ROCK co-injected oocytes and using oocytes from the same frog. Fasudil (10 μM), Y27632 (10 μM), MG132 (10 μM), chloroquine (200 μM), blebbistatin (10 μM), befreldinA (10 μg/ml) and dynasore (80 μM) were added to the survival medium 5–24 h before recordings, and also added to the recording solution.

2.2. Oocyte current recordings

For oocytes, whole-cell Ba^{2+} currents were recorded 2–5 days after injection under two-electrode voltage-clamp using a GeneClamp 500 amplifier (Axon Instruments, Union City, Ca) and a Ba^{2+}-containing external solution (in mM): BaOH, 10; TEAOH, 20; NMDG, 50; CsOH, 2; HEPES, 10; pH 7.2 with methanesulfonic acid. Current and voltage electrodes (less than 1 MΩ) were filled with: 3 M KCl, pH = 7.2 with KOH. Currents were filtered (500 Hz) and digitized (2 kHz) using a Digidata-1200 interface (Axon Instruments), and the pClamp software (v 7.01, Axon Instruments). Around 10–30 nl of BAPTA (in mM: BAPTA free acid, 100; CsOH, 10; HEPES, 10; pH 7.2 with CsOH) were injected into each oocyte (10 psi, 150 ms) at the beginning of the recording using a third electrode. Ba^{2+} currents were recorded during a 400 ms test pulse from −80 mV to +10 mV. Current analysis was performed using Clampfit ver. 10 (Axon Instruments). Current amplitudes were measured at the peak of the current. Isochronal inactivation curves (2.5 s of conditioning voltage followed by a 400 ms test pulse to +10 mV) were fitted using the following equation:

$$I/I_{max} = R_{in} + (1 - R_{in})/(1 + \exp((V - V_{in})/k_{in}))$$

where I is the current amplitude measured during the test pulse to +10 mV for conditioning voltage steps V varying from −80 to +50 mV; I_{max}: the current amplitude measured during the test-pulse for a conditioning step to −80 mV; V_{in}: the potential for half-inactivation; k_{in}: the slope factor and R_{in} the proportion of non-inactivating current. Current to voltage curves were fitted using the following equation:

$$I/I_{max} = G^*(V - E_{rev})/(1 + \exp((V - V_{act})/k))$$

where I is the current amplitude measured during depolarizations V varying from −80 to +50 mV; *Imax*: the peak current amplitude measured at the maximum of the current–voltage curve; G: the normalized macroscopic conductance; E: the apparent extrapolated reversal potential, V_t: the potential for half-activation; and k a slope factor.

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